The scent of brood recognition in *Formica* ants

UNNI PULLIAINEN

Evolution, Sociality and Behaviour
Organismal and Evolutionary Biology Research Program
Faculty of Biological and Environmental Sciences
University of Helsinki
Finland

ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki in Auditorium 1041, Biocenter 2 (Viikinkaari 5), on 22 of November 2019 at 12.00 o'clock noon.

HELSINKI 2019

SUPERVISED BY: **Prof. Liselotte Sundström**
Organismal and Evolutionary Biology research programme, Faculty of Biological and Environmental sciences, University of Helsinki

Dr. Nick Bos
Department of Biology, Faculty of Sciences, University of Copenhagen

Dr. Eva Schultner
Zoology and Evolutionary Biology, University of Regensburg

REVIEWED BY: **Dr. Carita Lindsted-Kareksela**
Department of Biological and Environmental Science, University of Jyväskylä

Dr. Joel Meunier
Institut de Recherche sur la Biologie de l'Insecte, University de Tours

EXAMINED BY: **Prof. Elli Leadbeater**
Department of Biological Sciences, Royal Holloway, University of London

CUSTOS: **Prof. Liselotte Sundström**
Organismal and Evolutionary Biology research programme, Faculty of Biological and Environmental sciences, University of Helsinki

MEMBERS OF THE THESIS ADVISORY COMMITTEE: **Dr. Heikki Hirvonen**
Organismal and Evolutionary Biology research programme, Faculty of Biological and Environmental sciences, University of Helsinki

Prof. Leena Lindström
Department of Biological and Environmental Science,
University of Jyväskylä

Dr. Saskya van Nouhuys
Department of Entomology, Cornell University, Ithaca, NY

ISBN 978-951-51-5607-5 (paperback)
ISBN 978-951-51-5608-2 (PDF)
http://ethesis.helsinki.fi
Unigrafia
Helsinki 2019
The Faculty of Biological and Environmental Sciences uses the Urkund system (plagiarism recognition) to examine all doctoral dissertations.

The ants, the ants
on tips of plants
on sticks, on stones,
on ice cream cones;

beneath the ground
they ebb and flow,
precisely know
who's friend, who's foe.

They dig, they climb,
they drag, they haul
(they never seem
to play at all).

At obstacles
thrown in their path
they laugh! (Well, really,
ants don't laugh:

they just speed up
their ant-like flow
and find a different
way to go.)

But when a gang
attacks their nest?
They beat their legs
against their chests,

they snap their
giant mandibles
and drive them out
with great success.

And then, after
the fight has quit,
they go back home
to baby-sit.

Joyce Sidman

TABLE OF CONTENTS

LIST OF THESIS CHAPTERS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals.

I Pulliainen, U., Helanterä, H., Sundström, L., Schultner, E. 2019: The possible role of ant larvae in the defense against social parasites –Proceedings of the Royal Society B. 286: 20182867.

II Pulliainen, U., Morandin, C., Bos, N., Sundström, L., Schultner, E.: The effect of social environment on sensory gene expression in ant larvae –*manuscript.*

III Pulliainen, U., Bos, N., d'Ettorre, P., Sundström, L. 2018: Caste-dependent brood retrieval by workers in the ant *Formica exsecta* - Animal Behaviour 140:151-159.

IV Pulliainen, U., Bos, N., d'Ettorre, P., Sundström, L.: Caste differences in surface hydrocarbons of brood in *Formica exsecta –manuscript.*

CONTRIBUTIONS

	Original idea	Methods	Data collection	Data analyses	Manuscript preparation
I	UP/ES	UP/ES	UP/ES/HH	UP/ES	UP/ES/LS/HH
II	ES/UP	ES/CM/UP	UP	CM	UP/ES/CM/LS/NB
III	UP/LS	UP/NB/LS/PdE	UP/NB	UP/NB	UP/NB/LS/PdE
IV	UP	UP/NB	UP	UP/NB	UP/NB/LS/PdE

CM: Claire Morandin
ES: Eva Schultner
HH: Heikki Helanterä
LS: Liselotte Sundström
NB: Nick Bos
PdE: Patrizia d'Ettorre
UP: Unni Pulliainen

ABSTRACT

Ants are an ecologically dominant group often acting as keystone species. The key to their success is their social lifestyle. Living in a society means that it is vital to distinguish a friend from a foe, in order to protect the colony and its resources from exploitation by unwanted quests. Recognition in ants is based on detecting a mixture of hydrocarbons found on the surface of other individuals. Typical colony intruders are adult individuals, and thus recognition among adults is very well studied. However, brood is an important part of these societies, representing the reproductive efforts of the whole society, and accurate recognition of brood, and by brood, could be important in many different contexts, such as when a colony gets usurped by a social parasite.

In this thesis, I investigated brood recognition in two *Formica* ant species from different points of view. I used behavioural assays to study brood discrimination of adults and larvae and further explored the mechanisms underlying recognition behaviour. I found that brood discrimination can be affected by caste, colony and species of brood, and that brood discrimination behavior follows inclusive fitness predictions. I also characterized the surface chemistry of pupae, showing how a species with a simple chemical profile in adults, can have complex brood profiles, which potentially carry cues for recognition. Furthermore, I studied the so far completely unknown chemosensory biology of ant larvae, and showed that larvae have the molecular machinery to perceive their social environment and react to information gathered from their surroundings.

This thesis adds to the accumulating knowledge that immature stages of social insects are not merely passive bystanders, by suggesting that ant larvae may take part in colony defense against social parasites. I also take the first steps in figuring out how ant larvae sense the world around them, and provide a basis for more detailed studies on the sensory biology of developing social insects. By describing the surface chemistry of immature ants, I furthermore help advance our understanding of the information ants use to recognize each other, highlight the context dependency of brood discrimination, and suggest new avenues of exploration in the field of chemical ecology.

TIIVISTELMÄ

Muurahaiset ovat ekologisesti merkittävä eliöryhmä, sillä ne ovat usein avainlajin asemassa, ja täten vaikuttavat muihin ekosysteemin eliöihin. Muurahaisten valtaisa menestys perustuu siihen, että ne elävät yhteiskunnissa. Yhteiskunnan toiminnan kannalta on äärimmäisen tärkeää pystyä erottamaan yhteiskuntaan kuuluvat yksilöt vieraista. Muurahaisilla yksilöiden tunnistaminen perustuu näiden pintaa peittävien hiilivetyjen aistimiseen. Hiilivetyjen perusteella muurahaiset pystyvät tunnistamaan kohtaamiaan yksilöitä hyvinkin tarkasti ja näin yhteiskunnan jäseniä ja resursseja voidaan suojella tunkeilijoilta. Tunkeilijat ovat useimmiten aikuisia muurahaisia, työläisiä tai kuningattaria. Aikuisten välistä tunnistamista on tutkittu paljon. Yhteiskuntaan kuuluu kuitenkin aikuisten lisäksi tärkeänä osana myös jälkeläiset (munat, toukat ja kotelot), joiden tuotantoon on panostettu merkittävä määrä yhteiskunnan resursseja. Jälkeläisten tunnistaminen voi olla hyvin tärkeää monessa eri yhteydessä, kuten silloin, jos muurahaispesiä uhkaavat sosiaaliset loiset ovat vallanneet pesän ja pyrkivät täyttämään pesän omilla jälkeläisillään.

Väitöskirjassani tutkin jälkeläisten tunnistusta kahdella *Formica*-suvun muurahaislajilla. Käsittelin tunnistamista useasta näkökulmasta, selvittäen käyttäytymiskokeilla aikuisten työläisten ja toukkien käyttäytymistä muita jälkeläisiä kohtaan, sekä tutkien tunnistamiseen liittyviä molekulaarisia mekanismeja. Tutkimuksista kävi ilmi, että jälkeläisten hyväksyntään vaikuttaa näiden kasti ja alkuperä (saman lajin toinen pesä, tai eri laji). Tutkin myös jälkeläisten hiilivetyprofiileja ja osoitin kuinka jälkeläisten pintakemia voi olla aikuisiin verrattuna monipuolisempi. Tämän lisäksi tutkin toukkien kemiallista aistinbiologiaa, josta ei aiemmin tiedetty mitään. Osoitin, että muurahaistoukilla on edellytykset aistia ympäröivää maailmaa ja reagoida ympäristön ärsykkeisiin.

Tämä väitöskirja vahvistaa ajatusta siitä, että yhteiskuntahyönteisten jälkeläiset eivät ehkä olekaan pesän passiivisia sivustaseuraajia, vaan voivat osallistua pesän toimintaan ja jopa pesän puolustukseen sosiaalisia loisia vastaan. Avasin myös oven muurahaistoukkien aistinbiologian tutkimukselle, luoden pohjaa tarkemmalle jälkeläisten aistimisen tarkastelulle. Jälkeläisten pintahiilivetyjen kartoittaminen auttaa osaltaan hahmottamaan sitä, millaista informaatiota muurahaiset käyttävät tunnistaessa toisiaan.

SUMMARY

1 INTRODUCTION

1.1 COMMUNICATION AND RECOGNITION

Communication, the passing of information from sender to a receiver is vital to all organisms. Not only do individuals communicate with each other, but communication also takes place within an organism (de Sousa 2008). When communicating, individuals (or other entities) detect stimuli in each other. These stimuli can be visual, olfactory, tactile or auditory. If a stimulus is produced without the intention of another individual acting upon this, the stimulus is a cue. When the stimulus is produced with the intention of another acting upon this, and the stimulus gets modified in response, the stimulus has evolved into a signal (Bradbury and Vehrencamp 2001). Accordingly, recognition behaviour involves at least two individuals, one of which (sender) bears a cue or a signal, and one (receiver), that detects and compares the cue/signal to an internal template (Liebert and Starks 2004). Based on the match, the sender is classified as a group member or an outsider. This is the perception component of recognition (Mateo 2004), an internal neural detection process that classifies individuals. The action component of the process follows when the receiver acts upon this information by, for example, showing aggression or acceptance (Reeve 1989; Liebert and Starks 2004). Even though action follows from perception, recognition of individuals does not always lead to action, and discrimination can be context dependent (Downs and Ratnieks 2000; Chapuisat 2004; Bos et al. 2010; Nehring et al. 2011; Sturgis and Gordon 2012).

1.2 RECOGNITION IN ANTS IS BASED ON CHEMICAL COMMUNICATION

Recognition is especially important for social insects, such as ants. Ants are characterized by a division of labour into castes as well as division of reproduction. Since reproduction is divided and majority of individuals in a colony are altruistic and do not reproduce, they must gain fitness indirectly. Through directing their altruistic acts towards related individuals (kin recognition), they can gain inclusive fitness benefits (Hamilton 1964). Kin recognition (in the narrow sense) has been proven to exist (e.g. Hannonen and Sundström 2003), but in general it is not necessarily required in viscous populations, such as colonies, that are likely to consist of related individuals. Thus nest-mate recognition is at the core of these societies.

To this end, chemical communication is used extensively in ants, as in all social insects (Wyatt 2003, 2010; d'Ettorre and Hughes 2008; van Zweden and d'Ettorre 2010; d'Ettorre and Lenoir 2011; Sturgis and Gordon 2012; Tsutsui 2013). To recognize other individuals, they detect a signature mixture: a subset of molecules on each other's surface (van Zweden and d'Ettorre 2010; Wyatt 2010). The mixture consists of mostly hydrocarbons, which can be endogenous (i.e. produced by the individual) or environmentally and socially acquired (Gamboa 2004; Katzav-Gozansky et al. 2004; van Zweden et al. 2010; Wicker-Thomas and Chertemps 2010; Bos et al. 2011; Martin et al. 2013) and are mixed within the colony by food sharing and grooming (Boulay et al. 2000; Soroker and Hefetz 2000; Leboeuf et al. 2016), creating a *Gestalt* colony odour (Crozier and Dix 1979). These hydrocarbons originally evolved to prevent desiccation (Gibbs 1998), but function extensively in chemical communication (Martin and Drijfhout 2009a; Wicker-Thomas and Chertemps 2010). The quantitative and qualitative variation in hydrocarbon profiles of ants, allow species- and colony specific detection, as well as detection according to e.g. task, sex, fecundity, age and caste (e.g. Wagner et al. 1998; Dietemann et al. 2005; Martin and Drijfhout 2009b; van Zweden and d'Ettorre 2010; Martin et al. 2013; Kleeberg et al. 2017).

Ants detect these chemical compounds with a chemosensory system, including a range of proteins in the antennal sensilla that detect, bind and carry the odorant molecules. The olfactory stimuli are then perceived in the antennal lobes and the higher integration centres in the brain, and ultimately lead to behavioural responses (Hildebrand and Shepherd 1997; Guerrieri et al. 2005; Ozaki and Wada-Katsumata 2010). It was long believed that nest-mate recognition (see below) is based on long-term memory of hydrocarbons (Lenoir et al. 1999), but more recent work (Ozaki et al. 2005) suggests that the olfactory receptors in the antennae desensitize to familiar odours, and that updating of the nest-mate template happens at the receptor-level, rather than in higher brain regions. Alternatively, habituation to nest-mate odours could happen in the antennal lobes, where the information gathered from the receptors is first integrated (Leonhardt et al. 2007; Guerrieri et al. 2009; Bos and d'Ettorre 2012).

1.3 NEST-MATE RECOGNITION

One of the main functions of chemical communication in ant colonies is to distinguish nest-mates from intruders (Jaisson 1991; Sturgis and Gordon 2012). Several models for nest-mate recognition have been suggested. Sherman et al. (1997) proposed two models: one is based on including individuals that carry desirable stimuli (D-present model), and other on excluding individuals that carry undesirable stimuli (U-absent model). An updated, non-nest-mate recognition model, the U-present model, was later proposed (Guerrieri et al. 2009; van Zweden and d'Ettorre 2010). This model supports the idea that higher brain regions are not involved in nest-mate-recognition, but that detection of non-nest-mates would happen due to either habituated antennal lobes or desensitized antennae (Ozaki et al. 2005; Leonhardt et al. 2007). The model thus proposes detecting and discriminating against non-nest-mates (undesirable individuals), instead of detecting each encountered nest-mate (D-present model), or the absence of non-nest-mates (U-absent model).

In addition, Reeve et al. (1989) proposed adaptively shifting conspecific acceptance thresholds. According to Reeve, there are two factors that dictate the stringency of the acceptance threshold: 1) the fitness costs of making acceptance errors (analogous to statistical Type II error, i.e., accepting

individuals that are undesirable), and rejection errors (analogous to statistical Type I error, i.e., rejecting individuals that are desirable) and 2) the frequency of interactions with desirable and undesirable individuals. These models (as all models) are simplifications of reality, but have been shown to work well (e.g. Downs and Ratnieks 2000) and help visualize the link between perception and action components of nest-mate recognition processes.

1.4 BROOD RECOGNITION

Brood (eggs, larvae and pupae), even though often neglected, are an important part of ant societies, as brood play an important role in colony interactions (Schultner et al. 2017). Workers show directed behaviour towards brood (Wilson 1971), and even tending to the queen(s) is secondary to tending to the brood (Lenoir 1981). Brood represent the reproductive investment of the colony and, from an inclusive fitness point of view, brood recognition can be crucial in many different contexts, such as kin conflicts within colonies (Schultner and Pulliainen 2019).

Furthermore, recognition of non-nest-mate brood may be especially important in species targeted by social parasites (Schmid-Hempel 1995). Many ant species have evolved to parasitize one another; these social parasites include permanent inquilines, temporary social parasites and slave-makers (Buschinger 2009). Of these, temporary social parasites are perhaps the most malicious, since the parasite queen invades a host colony, kills the resident queen(s) and usurps the colony by producing her own offspring. If the parasite queen succeeds, the host colony loses its entire future reproductive output (Buschinger 2009). As the cost of hosting such a parasite is high, the host species should invest in defence mechanisms to discriminate against both the parasitic queen, and her offspring (Alloway 1990; Achenbach and Foitzik 2009; Chernenko et al. 2011, 2013; Grüter et al. 2017). Accordingly, ant populations that are common hosts to social parasites have evolved to detect con- and hetero-specific non-nest-mates more accurately than populations without this threat (Martin et al. 2011).

Brood recognition and discrimination have been studied to some extent, and overall, brood discrimination appears to be context-dependent (reviewed in Schultner and Pulliainen 2019). This variation in brood discrimination is most likely not due to lack of chemical information. Although the surface chemistry of brood has not been studied in the same detail as that of adults (van Zweden and d'Ettorre 2010; d'Ettorre and Lenoir 2011; Sturgis and Gordon 2012; Tsutsui 2013), the role of surface hydrocarbons in brood recognition is well established (e.g. Viana et al. 2001; Souza et al. 2006; Achenbach et al. 2010a; Helanterä and d'Ettorre 2014). Brood surface chemistry has not only been shown to display variation among species and colonies, but also according to viability, developmental stage, sex, caste, maternity, paternity and even colony social structure (Schultner and Pulliainen 2019).

Although knowledge on brood recognition and surface chemistry of brood is slowly accumulating, next to nothing is known about social insect brood as the receivers of chemical information. Social insect larvae are surrounded by complex social interactions, and participate and respond to the social environment (Urbani 1991; Cummings et al. 1999; Schultner et al. 2013, 2017). But how do the immatures themselves (namely larvae) sense the world and individuals around them, and under what circumstances do they act on the perceived information?

2 AIMS OF THE THESIS

This thesis consists of four chapters, in which I explore brood recognition abilities of adults and larvae, and from there, take a step further into the world of chemical communication that is at the core of all recognition behaviour (Figure 1). In *Chapter I*, I consider the recognition abilities of ant larvae and ask whether they can recognize the origins of eggs. Here I suggest that ant larvae could act in nest defense against social parasites. In *Chapter II*, I characterize the so far completely unknown chemosensory biology of ant larvae and investigate how ant larvae sense the world around them. In *Chapter III*, I ask whether workers can recognize and discriminate against pupae of different origins. In *Chapter IV*, I analyze the surface chemistry of pupae according to sex and caste, and ask whether pupae carry informative odour cues.

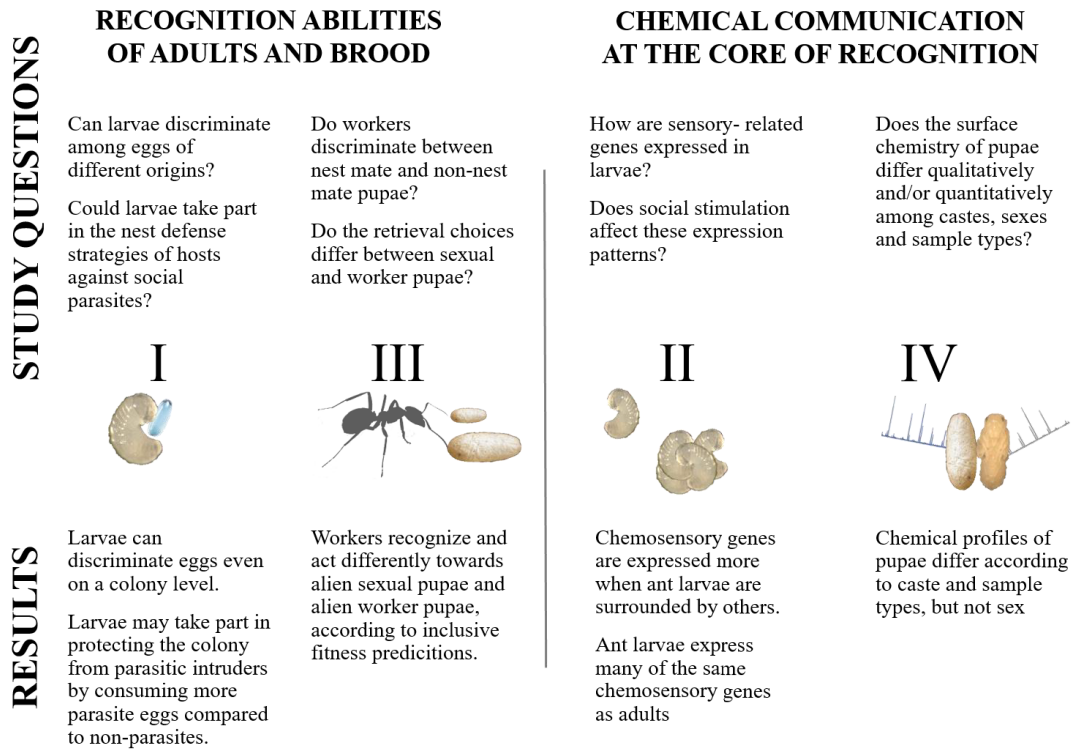


Figure 1) Study questions and main results of the four thesis chapters. (Photos ©Unni Pulliainen, ant: ©Nick Bos)

3 METHODS

3.1 THE STUDY SPECIES

In my thesis I used two species of *Formica* ants as the focal species: the common black ant, *Formica fusca* (*Chapters I* and *II*), and the narrow-headed ant, *Formica exsecta* (*Chapters III* and *IV*). *Formica fusca* is a common species in temperate forests (Collingwood 1979; Seifert 2007). It dominates the composition of ant fauna for several years after forest clearing, as it inhabits these semi-open habitats efficiently as a pioneer species (Punttila and Haila 1996). As the succession advances *F. fusca* nests are targeted and replaced by several temporary social parasite ant species (Punttila et al. 1991; Punttila and Haila 1996). Perhaps due to this high parasite pressure, both workers (Helanterä and Sundström 2007b; Helanterä and Ratnieks 2009; Chernenko et al. 2011, 2013; Helanterä et al. 2011, 2014; Martin et al. 2011) and larvae (Schultner et al. 2014; Pulliainen et al. 2019) exhibit precise discrimination abilities. Many ant species separate their brood according to developmental stage (Franks and Sendova-Franks 1992). *Formica fusca*, however, keeps their brood in piles, which allows larvae to access eggs within the nest. This makes the species ideal to study the recognition and egg consumption behaviour of larvae, especially in the context of social parasitism.

Formica exsecta in turn is found in sunny patches in mixed and dry woodland, where it inhabits forest clearings and borders (Czechowski et al. 2002). The population I used in experiments for *Chapters III* and *IV*, has been studied extensively with respect to different aspects of its ecology and genetics (e.g. Sundström et al. 1996; Vitikainen et al. 2011), as well as nest-mate recognition and the surface chemistry (Martin and Drijfhout 2009b; Martin et al. 2012a,b, 2013).

These characteristics make both of these species good representatives for studying brood recognition. Specifically, *F. fusca* is an excellent model to study potential defense strategies of hosts against temporary social parasites, and the chemosensory biology of larvae. The population of *F. exsecta* I used is ideal for studying variation in surface chemistry, and recognition behavior. In addition to the two focal species, I also used two social parasite species (*F. pressilabris* and *F. truncorum*) and two non-parasite species (*F. lemani* and *F. cinerea*) for egg production in *Chapter I* and *F. pressilabris* in *Chapter II*.

3.2 COLLECTION AND HOUSING

All of these species of ants can be found in the vicinity of Tvärminne Zoological station in southwestern Finland (with the exception of *F. lemani*, which I collected from Hyytiälä, central Finland). I collected pupae and adult workers from the monogyne (one reproductive queen) colonies of *F. exsecta* on the islands in the Tvärminne archipelago just outside of the Hanko peninsula (59°50' N, 23°15' E) (Figure 2), and entire colonies of *F. fusca* with workers and varying numbers of queens from the mainland (mainly around 59°90' N 23°25' E). Queens with workers from *F. pressilabris, F. truncorum* and *F. cinerea* were also collected from around the Tvärminne Zoological station.

The colonies (*F. fusca*, n=28 for *Chapter I*; n=6 for *Chapter II*) and colony fragments (*F. exsecta,* n=35 for *Chapters III* and *IV*) were sometimes further divided into smaller fragments for the experiments, housed at room temperature in either plastic boxes or glass-roofed Y-tong (porous stone) nests, fed with honey-egg-agar mix (Bhatkar diet, Bhatkar and Whitcomb 1970), and the nests were watered daily. Queens and workers from the additional species (*Chapter I*) were kept in the dark at +4ºC in the laboratory, until the onset of the experiments when queens were moved to individual Petri dishes to lay eggs.

Ethical note

The methods I used adhere to the ABS/ASAB guidelines for ethical treatment of animals in research. No rules govern the treatment of our study organisms. To ensure minimal disturbance to ant colonies, only a fraction of workers, queens and brood was collected, where possible. Ants were fed and the colonies were watered daily, and individuals were treated carefully.

3.3 BEHAVIOURAL ASSAYS AND EXPERIMENTAL SET-UPS

Behavioural experiments are the backbone of this thesis. *Chapters I* and *III*, which lay the groundwork for *Chapters II* and *IV*, rely heavily on behavioural assays. In *Chapter I*, I used an egg consumption assay to find out whether larvae of the common social parasite host species, *F. fusca*, consume eggs differently, depending on the origin of the queen that laid the eggs (parasite, non-parasite, nest-mate, or conspecific non-nest-mate). Since *Formica* ant larvae do not move considerably, I gave the larvae easy access to an egg by placing them on top of it in a Petri dish. Egg consumption was documented after 48 hours (Figure 3).

To study larval sensory gene expression in different social environments (*Chapter II*), I placed young *F. fusca* larvae (1-3 days post hatching) in a Petri dish for 24 hours, either alone (social isolation) or with other nest mate larvae and eggs (social stimulation) (Figure 4). Larvae were then stored for RNA extractions. Finally, to measure the propensity of *F. exsecta* workers to retrieve pupae depending on the nest of origin (nest mate vs. non-nest mate), I used a pupa retrieval assay (*Chapter III*). I conducted two sets of tests, one with worker pupae and other with sexual pupae (future reproductives). In both sets I placed an arena on top of a nest box, which contained workers. On the arena, I placed nest mate and non-nest mate pupae. I allowed the workers to retrieve the pupae from the arena, and recorded the origin and order of pupae brought to the nest box (Figure 5).

.

Figure 2) Image of *F. exsecta* collection on the island of Furuskär in Tvärminne archipelago. © *Siiri Fuchs*

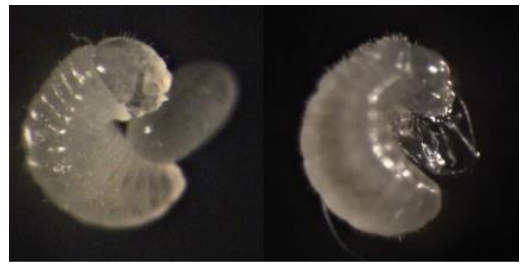


Figure 3) *Formica fusca* larva consuming *F. pressilabris* egg, with the remains of the eggshell in the picture on the right. © Proceedings of the Royal Society

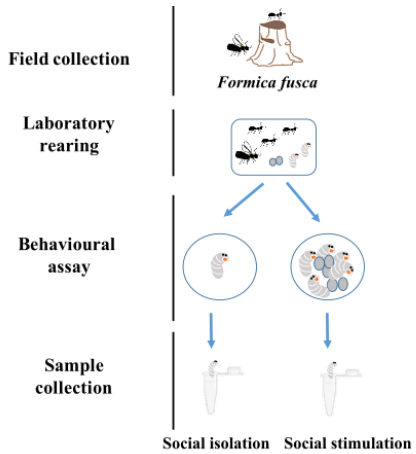


Figure 4) Flowchart of the setup for RNA-sequencing in *Chapter II*. ©2019 Springer Nature Publishing

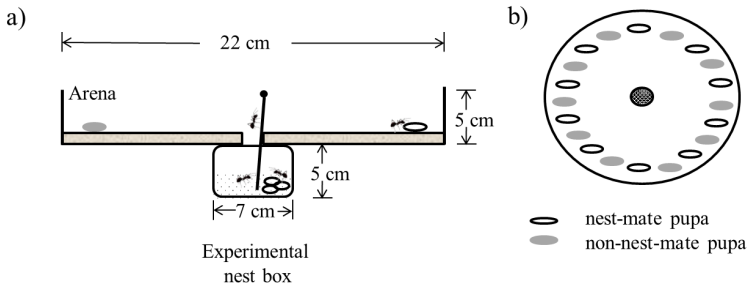


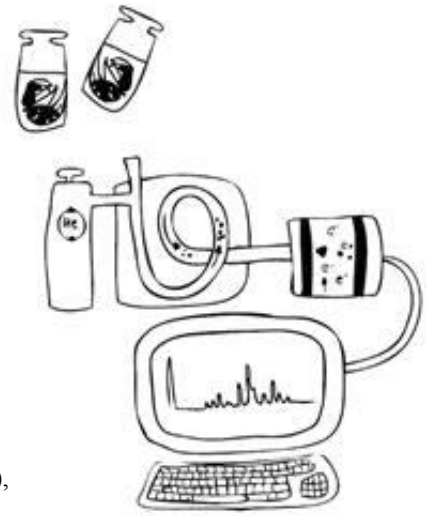
Figure 5) The pupae retrieval set-up. a) Side view of the set-up with a hole in the middle of the arena to allow access from the nest box. b) Top view of the arena with alternating nest-mate and non-nest mate pupae placed in a circle (Rosengren and Cherix 1981; adapted from Maeder et al. 2005). © The Association for the Study of Animal Behaviour

3.4 CHEMICAL ANALYSIS

Chemical analysis of surface hydrocarbons is central to studying chemical communication and recognition behaviour in social insects (van Zweden and d'Ettorre 2010). I used chemical analysis to reveal between-colony differences in surface chemistry of pupae (*Chapter III*) and to explore how chemical distance between eggs of different species may influence larval egg consumption behaviour (*Chapter I*). I furthermore characterized the quantitative and qualitative differences of the surface chemistry of sexual and worker pupae, as well as adult workers of *F. exsecta* (*Chapter IV*). In order to reveal potential differences in compounds between cocoons and the developing individuals within the cocoon, I analyzed their chemistry separately. All the analyses of surface chemicals were performed with gas chromatography, coupled with mass spectrometry (GC-MS, Box 1). Surface chemicals were extracted by first immersing the samples in pentane, which extracts the layer of surface chemicals to the liquid. Pentane was then moved to a separate vial and allowed to evaporate. The surface chemicals left in the bottom of the vial were rediluted in pentane and injected into a GC-MS (Box 1). The hydrocarbons in each sample were then identified using their retention times and the mass spectra and their relative amount quantified.

Box 1) GC-MS

Gas chromatography (GC) and mass spectrometry (MS) are microanalytical techniques used concurrently to identify and quantify chemical compounds that exist in a mixture, and are commonly used in the analysis of surface chemistry.



Gas chromatography

Samples dissolved in a solvent are injected to an inlet and vapourized into a gas. This gas is then carried through a column towards a detector. As the column is heated up slowly, the different compounds migrate through the column at different temperatures according to their volatility. Different compounds thus reach the detector at different times (retention time) and can be later detected as separate peaks in a graph (chromatogram), positioned according to their retention times.

Mass spectrometry

In the mass-spectrometer, each separated compound is broken into electrically charged fragments of different size, due to a beam of electrons being passed through these molecules. These fragments travel through a magnetic tunnel and are sorted according to their mass to charge ratio (m/z) and their relative abundance, which are shown in a graph (mass spectrum). The mass spectrums of chemical compounds do not change, hence the mass spectra can be used to identify the different compounds. Compounds tend to break apart in specific ways, and thus by analyzing all the fragments and their abundance, one can infer the original molecular structure.

Both retention time from the GC, and the mass spectrum from the MS, are used in identifying compounds in a sample. Quantification in turn is done by measuring the relative surface area of each peak in a chromatogram.

References:

Sparkman, O. Penton, F. Kitson, O. Sparkman, Z. Penton, and Kitson, F. 2011. Introduction and History. Gas Chromatogr. Mass Spectrom. 2–13. Academic Press.

3.5 GENETIC ANALYSIS

Determination of larval sex

Male *Formica* larvae have been shown to cannibalize more than females (Schultner et al. 2013, 2014). Thus, to avoid a possible bias in the results of the egg consumption experiments (*Chapter I*), it was important to determine the sex of larvae involved and use it as a variable in the analysis. For this I genotyped larvae from each colony. Their DNA was extracted with Chelex and Proteinase-K and samples were amplified at 8 microsatellite loci previously tested in this species (Schultner et al. 2014) using the

QIAGEN Type it Microsatellite Multiplex protocol and analyzed with an ABI sequencer. Microsatellite peaks were scored using Genemapper, and since hymenopteran females are diploid and males are haploid, the sex of larvae were determined based on heterozygosity/homozygosity of these loci.

RNA sequencing

To characterize the sensory-related gene expression patterns in *F. fusca* larvae, and to identify candidate genes involved in sensory perception, I compared gene expression of larvae from two social environments (social environment vs isolation) and characterized expression patterns of candidate sensory-related genes (*Chapter II*). To explore gene expression, I used RNA sequencing on RNA that was extracted from the whole body of larvae.

4 MAIN RESULTS AND THEIR INTERPRETATION

I showed that brood discrimination in two *Formica* ant species is context-dependent and can be affected by the colony origin (*Chapter I* & *Chapter III*), caste (*Chapter III*), and/or species (parasite versus non-parasite) (*Chapter I*) of brood (Figure 1). Furthermore, the results of *Chapter I* and *Chapter III* indicate that discriminatory decisions are made in accordance to predicted inclusive fitness benefits. Characterization of the chemical profiles of *F. exsecta* pupae in *Chapter IV* highlights the diversity of surface chemistry in developing ants. Finally, the results of *Chapter III* provide first insight into chemosensory gene expression in *F. fusca* larvae and offer support for the idea that larvae can detect changes in their social environment. Below I discuss these results and interpret their significance in more detail.

4.1 BROOD DISCRIMINATION BEHAVIOUR OF ADULTS AND LARVAE

Larvae as discriminators

In *Chapter I*, I showed how larvae of a common host species to social parasites (*F. fusca*) consume social parasite eggs more than eggs of non-parasitic species. Furthermore, larvae consumed non-nest-mate eggs, but no nest-mate eggs (Figure 6). This indicates that *F. fusca* larvae have the ability for accurate nest-mate recognition, just like adults of this species (Hannonen and Sundström 2003; Helanterä and Sundström 2007a; Helanterä and Ratnieks 2009; Chernenko et al. 2011, 2013; Helanterä et al. 2011; Martin et al. 2011), and that they can assess whether the encountered species is a parasite or not. *Formica fusca* adults have been suggested to have acquired accurate recognition abilities as a response to parasite pressure in areas where it is commonly parasitized (Martin et al. 2011). Larval recognition abilities shown here may also result from parasite pressure.

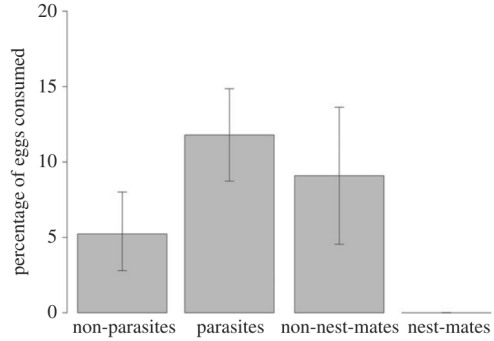


Figure 6) Average rates of egg consumption by *F. fusca* larvae, when offered non-parasite eggs (n = 287), parasite eggs (n = 424), non-nest-mate eggs (n = 154), or nest-mate eggs (n = 56), with 2.5 and 97.5% quantiles. © Proceedings of the Royal Society

Previous studies on larval recognition abilities in the context of egg consumption have shown that larvae can adjust their consumption behavior according to traits such as egg viability and colony origin (Baroni Urbani 1991; Crespi 1992; Schultner et al. 2013, 2014). Although not studied in great detail, the fact that larvae can recognize other individuals is not surprising, since pre-imaginal learning (learning during the larval stage) affects adult recognition abilities in several species of ants (Isingrini et al. 1985; Hare and Alloway 1987; Carlin and Schwartz 1989; Signorotti et al. 2013).

That larvae prefer to consume social parasite eggs suggests that they may help the colony defend against social parasites. Although it is not known at which stage of the parasite attack the host queen is executed, the resident queen has most likely been killed at the time the parasite queen lays her eggs. So how can destruction of parasite offspring still be beneficial? There are several ways in which a host colony can gain fitness benefits, even in the absence of their queen(s). First, host workers can produce unfertilized eggs (Helanterä and Sundström 2005, 2007b; Chernenko et al. 2011, 2013; Grüter et al. 2017), which grow into males. Second, larvae can increase their own survival by consuming eggs (Schultner et al. 2013). As an added advantage, female larvae are more likely to develop into a queen in the absence of a mature queen, which could lead to direct fitness benefits (Helanterä and Sundström 2005). This study is the first to show that ant larvae can participate in defensive acts, which benefit the colony as a whole.

Nest-mate recognition

In *Chapter III*, I showed how adult *F. exsecta* workers retrieve nest-mate and non-nest-mate pupae differently depending on the caste of the pupae. Nest-mate sexual pupae (future males and gynes) were preferred over non-nest-mates (Figure 7a). However, worker pupae retrieval was not affected by the colony origin of pupae, as both nest-mate and non-nest-mate pupae were retrieved at similar rates (Figure 7b).

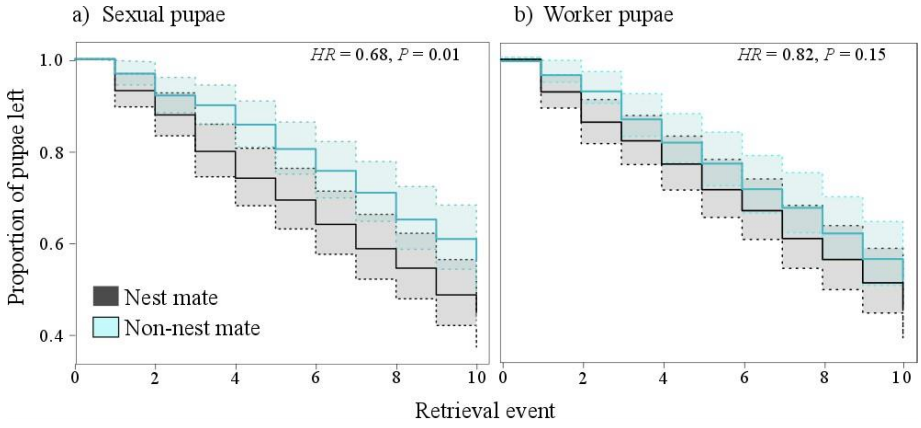


Figure 7) Proportions of nest-mate and non-nest mate a) sexual pupae and b) worker pupae not retrieved during the first 10 retrieval events, including the 95% confidence intervals indicated by the dotted lines and shaded areas. © The Association for the Study of Animal Behaviour

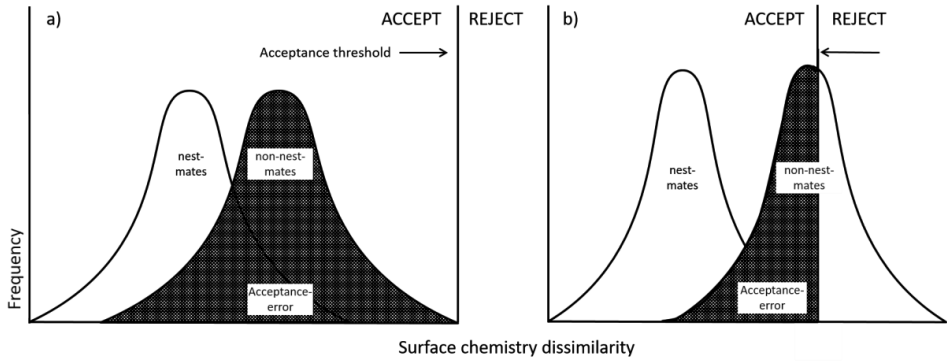


Figure 8) Schematic visualization of theoretical frequency distributions of the nest-mate and non-nest-mate surface chemistry for worker pupae (a) and sexual pupae (b). The figures also show the assumed position of the acceptance threshold, as well as the acceptance-error region this threshold creates (Adapted from Reeve 1989). Even though the rationale for non-linear acceptance thresholds is well supported (van Zweden and d'Ettorre 2010), for simplicity I present linear thresholds here.

The results appear to follow predicted fitness consequences related to the rejection- and acceptance errors, which in the case of pupa retrieval, differ between discrimination of sexual and worker pupae. In the case of worker pupa retrieval, the cost of making acceptance errors is minimal, since alien worker pupae do not pose a threat to a colony, and can even be beneficial by acting as additional work force once eclosed (Buschinger et al. 1980; Isingrini et al. 1985; Fénéron and Jaisson 1995; Orivel et al. 1997). There is also no cost of rearing these non-nest-mates at this developmental stage, since pupae do not need to be fed or require as much tending as earlier life stages. This creates a universal acceptance threshold, since there are no costs related to acceptance errors (Figure 8a).

Accepting non-nest-mate sexual pupae however, which do not contribute to the workforce, use a high amount of resources, and could potentially reproduce in the colony, results in higher costs of acceptance errors. Furthermore, and probably more importantly, rejecting nest-mate sexual brood (rejection error) should be avoided, as sexual offspring represent the fitness of the colony. Thus, the fitness costs related to both the acceptance and rejection errors push the acceptance threshold towards a more stringent threshold. However, according to Reeve's (1989) optimal-acceptance-threshold –theory, the acceptance threshold is not only affected by the fitness consequences of these errors, but also by the frequency of encounters with nest-mates and non-nest-mates. The optimal acceptance thresholds become more permissive as the relative frequency of interaction with desirable recipients increases. Hence, as the frequency of encountering non-nest-mate sexual pupae (as well as worker pupae) is presumably low, and the cost of potential rejection error is high, the threshold allows a proportion of acceptance errors (Figure 8b).

The same rationale can be used to discuss egg consumption choices of larvae (*Chapter I*). There was a significant difference in consumption of nest-mate eggs (0% of eggs consumed), compared to non-nest-mate eggs (9% of eggs consumed) (Figure 6). The acceptance of non-nest-mate eggs is potentially costly and the frequency of encountering parasite eggs in this species is high (Martin et al. 2011), which would lead to a stringent acceptance threshold.

However, also here the cost of rejection error, i.e. consuming siblings, is high, which pushes the optimal acceptance threshold to be more permissive and to ensure the inclusion of all nest-mates. Even though the parameters affecting the optimal threshold are not identical between egg consumption behaviour (*Chapter I*) and sexual pupae retrieval (*Chapter III*), in both cases minimizing rejection errors and costs related to acceptance errors are balanced and thus the optimal acceptance threshold can be assumed to be similar (Figure 8b).

4.2 MECHANISMS UNDERLYING RECOGNITION BEHAVIOUR

Genetic underpinnings of larval chemosensory biology

In *Chapter I*, I found that larvae prefer to consume social parasite eggs and can even discriminate eggs on a colony level. This discrimination is most likely based on chemical cues on the surface of eggs (Johnson et al. 2005; Schultner et al. 2013; Helanterä and d'Ettorre 2014; Helanterä et al. 2014). However, the chemosensory biology of ant larvae has not been studied in much detail. How do ant larvae sense the world around them? I addressed this question using a transcriptomic approach to search for expression of chemical communication related genes. In *Chapter II*, I subjected larvae of *F. fusca* to two social environments (isolation and stimulation with the presence of nest-mate larvae and eggs) and characterized the expression of sensory-related genes. I found that social stimulation affected sensory gene expression (Figure 9), and that genes overexpressed in stimulated larvae were related to chemosensory perception. The sensory genes overexpressed in socially stimulated, compared to isolated larvae, included genes from the major chemosensory gene families known to carry and bind odorant compounds in social insects (Ishida et al. 2002; Kulmuni and Havukainen 2013; McKenzie et al. 2014; Hojo et al. 2015). I also found other potentially interesting sensory genes, which have been shown to be involved in chemoreception and expressed in sensory organs in either adult insects and/or insect larvae (Sevala et al. 2000; Guntur et al. 2004; Willingham and Keil 2004; Fujikawa et al. 2006; Yoshizawa et al. 2011; Andrés et al. 2014). This is the first step in understanding chemosensory activity in ant larvae, with the results suggesting that ant larvae have the sensory capabilities to perceive their social environment.

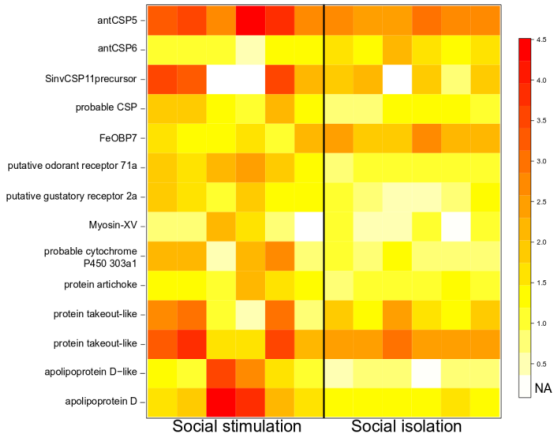


Figure 9) Heat map presenting expression values (logFPKM [fragments per kilobase million] +1,) for differentially expressed chemical communication-related and other sensory genes. Each column represents either a socially stimulated, or a socially isolated larva (*Chapter II*, Figure 5).

Surface hydrocarbons of F.exsecta

I found differences in the surface hydrocarbon profiles of the different sample sets of *F. exsecta*: adult workers, cocoons (gynes, males and workers), and developing individuals (gynes, males and workers). The proportions of different classes of hydrocarbons varied between the sample sets (Figure 10). All pupa samples had larger ratios of *n*-alkanes than adult workers, and correspondingly smaller quantities of (Z)-9-alkenes (Figure 10). Whether the *n*-alkanes have any brood recognition functions, or are simply more abundant in brood due to the lack of (Z)-9-alkenes, remains to be tested. The (Z)-9-alkenes are nearly missing from the developing individuals, which suggests that these nest-mate recognition compounds (Martin et al. 2008, 2013) are not produced by the developing individuals themselves. This supports the notion that nest-specific compounds can be acquired from the environment and other ants (Boulay et al. 2000; Soroker and Hefetz 2000; Katzav-Gozansky et al. 2004; Bos et al. 2011; Leboeuf et al. 2016) and the hypothesis that the colony *Gestalt* odour is acquired at a chemical integration period at the early adult stage (Lenoir et al. 1999).

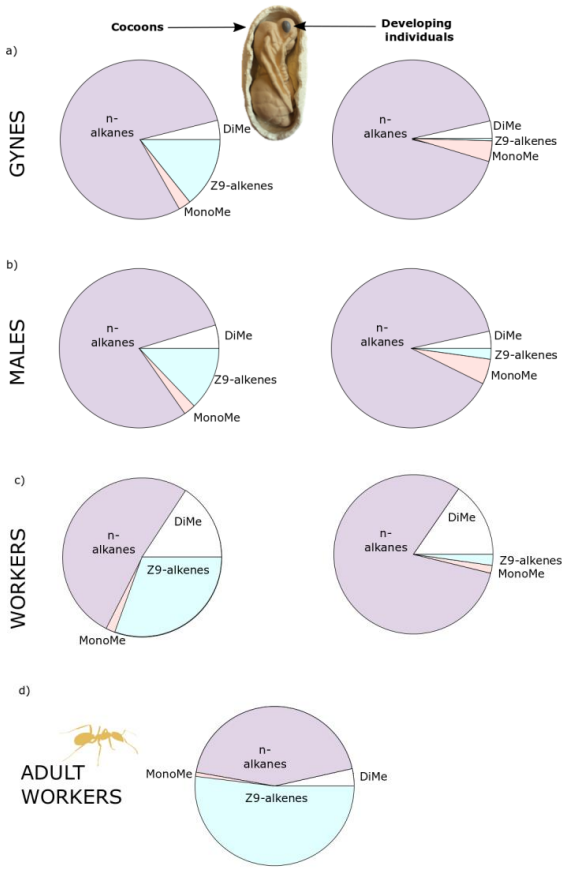
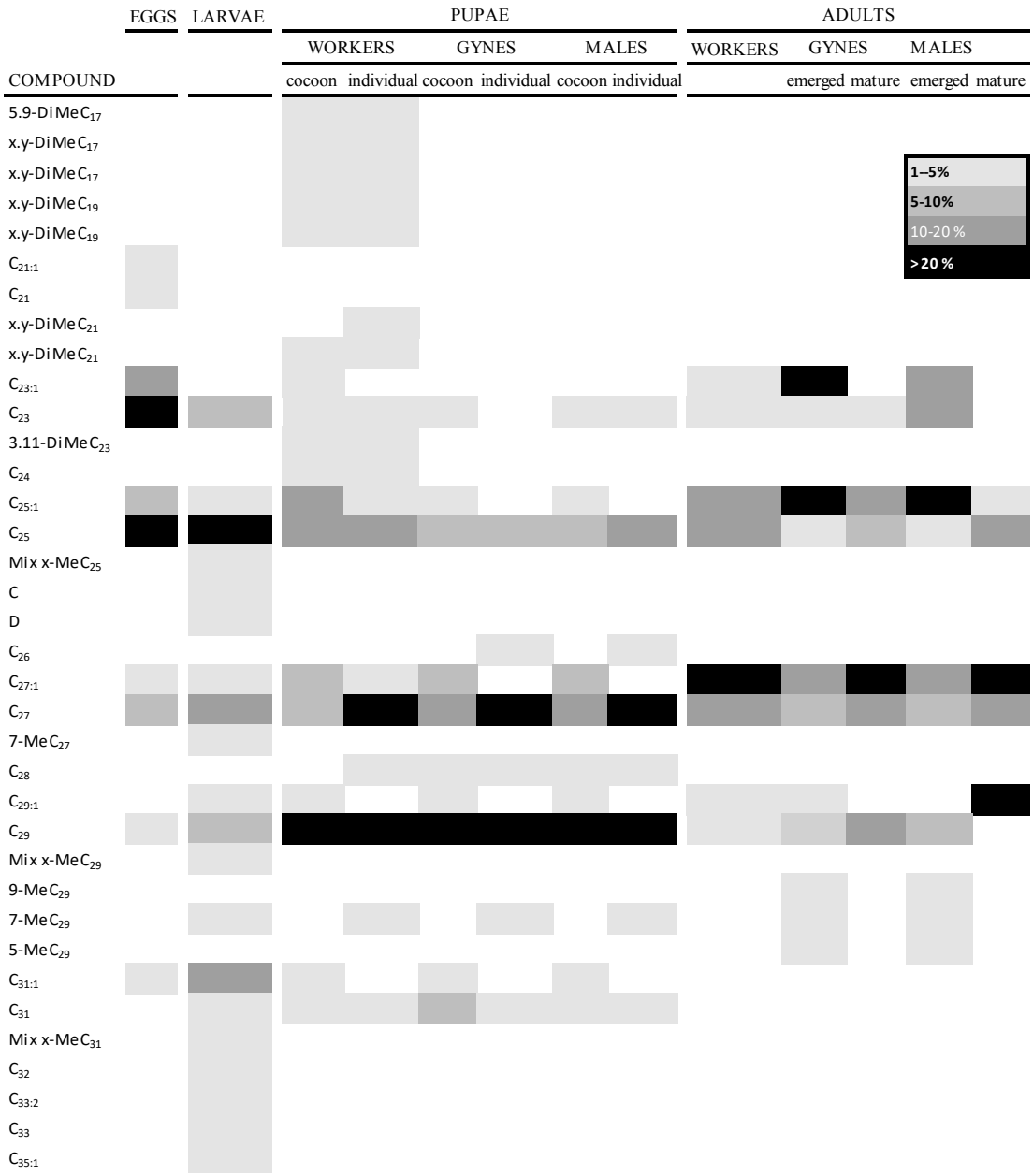


Figure 10) Relative representation of groups of hydrocarbons (linear *n*-alkanes, Z(9)-alkenes, monomethylated and dimethylated alkanes) in gyne (a), male (b) and worker (c) pupae, as well as adult workers (d). Photo ©Unni Pulliainen

Table 1) Heat map of compounds that have on average >1% representation in the different developmental stages of *F. exsecta*: eggs (Helanterä and d'Ettorre 2014), larvae (Peignier et al. 2019), pupae and adult workers (*Chapter IV*), emerged and mature sexuals (Martin et al. 2014). The % of compounds in emerged and mature sexuals was estimated from Figure 1 in Martin et al. (2014), (*Chapter IV,* Table S2).



I confirmed that adult workers of *F. exsecta* have, compared to other ant species, an extremely simple cuticular hydrocarbon profile (Table 1) (Martin et al. 2008, 2012a, 2013; Martin and Drijfhout 2009b). Contrary to what has been found in other species of ants (Bagnères et al. 1991; Akino et al. 1999; Viana et al. 2001; Elmes et al. 2002; Souza et al. 2006; Richard et al. 2007; Fouks et al. 2011; Helanterä and d'Ettorre 2014), the chemical profiles of brood in *F. exsecta* are more complex (Table 1). The adult profiles, in both workers, as well as mature sexuals (Martin et al. 2014) consist of a few dominating compounds (*n*-alkanes and their (Z)-9-alkene counterparts), whereas the brood profiles have a more equal representation of a larger selection of compounds, including methylated alkanes.

Of these brood-specific compounds, there were a few that could potentially be used in a brood recognition context. These include the longer-chained hydrocarbons: (Z)-9-alkene $C_{31:1}$ and n-alkane C_{31}, which are found in very low proportions, or not at all, in adult workers, newly emerged and mature sexuals. Both are found in larvae, and $C_{31:1}$ is also found in eggs (Table 1). Another example of a potentially interesting brood-specific compound is the monomethyl 7-MeC_{29}, which in my data was present in developing individuals in moderate quantities, and present only in trace quantities in the remaining sample sets. The 7-MeC_{29} was also found in larvae, and newly emerged sexuals, but almost undetectable in matured sexuals and adult workers (Table 1). This suggests that the compound is most likely synthesized by the developing individuals, starting at the larval stage. Thus, the compound may for instance signal the need for care. Monomethylated compounds have the potential to act as pheromones, as for instance, monomethylated alkane 3-MeC_{31} plays a role in queen-worker communication and suppresses worker ovarian activation in *Lasius* ants (Holman et al. 2010).

In *Chapter III*, I found that workers discriminate against nest-mate and non-nest-mate pupae differently depending on the caste of the pupae. This is most likely not due to workers not being able to recognize the origins of worker pupae, since both worker and sexual pupae carried colony information. I did not give workers a chance to choose between sexual and worker pupae, hence, it remains to be tested whether they can discriminate between these castes. There was a difference in the short-chained dimethyl hydrocarbons between sexual and worker brood (Table 1), (*Chapter IV*), but the role of these compounds as recognition cues remains to be tested. There are only few studies with evidence

of caste-specific brood recognition in ants (Brian 1975; Edwards 1991; Achenbach et al. 2010; Fox et al. 2011; Villalta et al. 2016; Penick and Liebig 2017), and in some of these, discrimination has been suggested to be based on a mixture of cues, including chemical and tactile cues such as size differences and pilosity (i.e. hairiness).

5 CONCLUSIONS

I showed that social insect larvae have the molecular machinery to perceive their social environment (*Chapter II*) and react to information gathered from their surroundings (*Chapter I*). I also showed how brood nest-mate discrimination is context dependent and that the acceptance threshold is shifted according to fitness predictions. The optimal response can be to reject non-nest-mates in one context and accept them in another. Although the context of nest-mate recognition is different in pupae retrieval assays (*Chapter III*) and larval egg consumption assays (*Chapter I*), the same principles of nest-mate discrimination apply. Furthermore, by characterizing the surface chemistry of *F. exsecta (Chapter IV)*, I built on the existing knowledge available for other developmental stages of this species, thus highlighting the diversity of surface chemistry in social insects across developmental stages, and suggesting possible differences in origins of hydrocarbons in different developmental stages.

This thesis helps advance the field by adding to the accumulating knowledge that immature stages of social insects are not merely passive bystanders (Schultner et al. 2017), but can participate in colony life. I contribute the first study (*Chapter I*) to suggest ant larvae take part in colony defense against social parasites by trying to eliminate eggs laid by intruding queens. I also take the first steps in describing how ant larvae sense the world around them, and provide a basis for more detailed studies on the sensory biology of developing social insects (*Chapter II*). By describing the surface chemistry of immature ants (*Chapter IV*), I help advance our understanding of the information available for recognition, highlight the context dependency of brood discrimination in ants, and suggest new avenues of exploration in the field of chemical ecology (*Chapter I*).

6 ACKNOWLEDGEMENTS

I'll start by saying 'thank you' to
the person who introduced me to
the wonderful world of ants and evolution.
***Lotta,** without you*
there would be no conclusion,
no start, no end, to this ant-journey of mine,
which started with curiosity,
and now, 12 years later,
(maybe) ends with this rhyme.

From you I learned so very much,
that I find it hard to express
all my gratitude for your patience and trust,
and belief in my success.
But here it is in its' simplicity:
A heartfelt 'thank you' for investing in me!

Nick Bos,
my boss,
one of the most brilliant minds
I've ever met.
If I needed to name someone,
one person that I would place my bet,
to solve a problem big or small,
I would have no doubt at all.
For sure I know I would pick you,
as you always know just what to do.

I wouldn't have had a clue how to do
so many things that to you
are just like breathing,
and to you I owe a lot of my succeeding.

The third person that sat by my side
when writing this work
is someone who I call a dearest of friends,
someone whom without
I might have gone berserk.
***Eva** my dear,*
there are so many memories
I keep close to my heart,
that honestly I don't know where to start!

There were the electric larvae
and the nights in the rocks,
the endless conversations
that made me understand myself better,
you are my personal
and scientific trendsetter,
like an awesome-o gift
that always knocks at my door
at exactly the right time.
You, my love,
I wouldn't exchange for any dime.

***Heikki**, dear Heikki,*
You are the calmest dude,
so amazingly chill,
I simply love your company,
that's the drill.
Thank you also for being
such a massive science nerd
and the bestest colleague.
Oulu is lucky to have you,
while the rest of the world is in need.

***Patrizia**, thank you*
for all the trips in Paris,
for introducing me
to the smelly world of ants.
It was such a pleasure
to work and write with you,
so to you too the biggest of thanks!

*Thank you also to **Claire***
for our collaboration.
I don't think I would have managed
those papers without you,
you are a part of this celebration.

***Dalial**, I'm sad we didn't*
become coworkers,
but I'm happy to call you a friend,
I hope this is a relationship
that doesn't have an end!
*And **Rose**, thank you for hiring me*
for a brief little time,
it would have been such a great pleasure
to continue working with you,
there is much more fun to be had,
so no point in feeling blue.

***Sanja**, I've learned so much from you,*
how everything you do is just..true.
You have been my compass
of truth and fair,
You put me in the spot and made me think.
You make me question and care.
I thank you for it.
I love you for it.

*And hey thank you **Jack***
for each and every shared snack,
the funny gifs
and the bad dad jokes,
you made me giggle,
thanks for that!
You are like the little brother I never had,
and it will be incredibly sad
not sharing a workplace with you,
so just going for lots of beers from now on
will have to do.

*All of **Team Antzz**, all of **ESB**,*
each and every one of you
are so special to me.
So many happy memories
we shared by the Baltic Sea,
and in Viikki,
sipping our coffee and tea.
Thank you for making my work
so much fun.
You were there for me always,
you were my sun!

Especially everyone who ever shared
*the office **5202**,*
seriously,
without you,
coming to work would have been so sad.
No matter if I felt under the weather
or mad,
you would pick me up.
I will always cherish
the good times that we had.

*For **Perttu**,*
*the **Pre-examiners***
*and the **Thesis advisory board**,*
thank you for all of your help
in allowing me to reach for
the hat and the sword!

*Thank you all the **colleagues***
***in the 5th floor**,*
thank you people in the lab,
I am truly so glad
to have been able to see you guys
by the coffee machine,
by the ABI machine,
by the copying machine,
and everywhere in between.

Your smiles and good morning's
were not left unnoticed,
you made my time at work warm and fun,
and I truly thank you for it
(except the someone
who always stole my food,
for you
I wish a continuous shitty mood).

*Thank you also **Kide-team**,*
for giving me an amazing new home.
Who would have known
that so quickly you'd become
so dear to me.
Your stupid humor and brilliant minds
create the best new job there can be!

*Thank you **friends**!*
All of you!
Kalismutsit,
Mitäh?
Lonkerokerho,
Vaeltajat,
Dancing buddies,
***My tribe** (the h**s on boats),*
for keeping me entertained and sane.
Thank you for allowing me
to blabber about ants,
and to talk about things in vain.

Thank you for your company,
your humor, and your wit,
without you life wouldn't be this much fun,
there wouldn't be so many giggles in it.
And to be honest,
to laugh is to live,
without joy, there isn't much to give.

*Thank you **Maiju** for being a friend*
and taking care of
both me and my daughter.
Although you are as efficient as two in one,
you could never be replaced
by anyone other.

*Crew **Savolainen**,*
I want to thank you for the warmest hugs,
thank you for the filled coffee mugs,
thank you for taking me in to your family,
thank you for allowing me to be me.

*Thank you **Family Pulliainen***
for allowing me to find my path on my own,
yet giving me the support and security,
thank you for believing in me.

*Thank you **dad** for always repeating*
"asioilla on taipumus järjestyä".
It has made me trust in this world,
and trust in myself too.
That is a huge thing to achieve,
to trust that the world carries you.

Mom,
thank you for teaching me
how to be a stable,
happy
and a quirky human too.
Your odd sense of humor is contagious,
I love you for being you.

Lastly, my little family:
***Kimmo** and **Tilda** -the mini-me.*
Kimmo, you are my rock,
my life is so safe with you,
thank you for always pushing me
to be a better person,
thank you for everything you do!

A huge thanks for allowing me
to have all of this time and support
the last few months,
and the previous fifteen years.
Thanks for hugging me in so tight
and thank you for wiping those tears.

Thank you for taking care of
the Ginger-Ninja,
the home,
and simply being you.
Thank you for giving me all that you have,
and for always being true.

Tilda, sie oot parasta
ja hassuinta
mitä tiedän.
Oot ihan huikea stressinlievittäjä,
siun ansiosta vaikeatkin päivät siedän.
Siun vuoksi asiat loksahti paikalleen
ja oikeisiin mittasuhteisiin meni.
Tää "äitin muurahaistutkimuskirja"
on omistettu sulle.
Ihan täydellistä että oot olemassa,
rakkain, tärkein, kultaseni!

7 REFERENCES

Achenbach, A., and S. Foitzik. 2009. First Evidence for Slave Rebellion: Enslaved Ant Workers Systematically Kill the Brood of Their Social Parasite *Protomognathus americanus*. Evolution. 63: 1068-1075.

Achenbach, A., V. Witte, and S. Foitzik. 2010. Brood exchange experiments and chemical analyses shed light on slave rebellion in ants. Behav. Ecol. 21:948–956.

Akino, T., J. J. Knapp, J. A. Thomas, and G. W. Elmes. 1999. Chemical mimicry and host specificity in the butterfly *Maculinea rebeli*, a social parasite of *Myrmica* ant colonies. Proc. R. Soc. B Biol. Sci. 266:1419–1426.

Alloway, T. M. 1990. Slave-species ant colonies recognize slavemakers as enemies. Anim. Behav. 39:1218–1220.

Andrés, M., E. Turiégano, M. C. Göpfert, I. Canal, and L. Torroja. 2014. The Extracellular Matrix Protein Artichoke Is Required for Integrity of Ciliated Mechanosensory and Chemosensory Organs in *Drosophila* Embryos. Genetics 196:1091–1102.

Bagnères, A.-G., E. D. Morgan, and J.-L. Clement. 1991. Species-specific secretions of the dufour glands of three species of formicine ants (Hymenoptera: Formicidae). Biochem. Syst. Ecol. 19:25–33.

Baroni Urbani, C. 1991. Indiscriminate oophagy by ant larvae: an explanation for brood serial organization? Insectes Soc. 38:229–239.

Bhatkar, A., and W. H. Whitcomb. 1970. Artificial Diet for Rearing Various Species of Ants. Florida Entomol. 53:229–232.

Bos, N., and P. d'Ettorre. 2012. Recognition of Social Identity in Ants. Front. Psychol. 3:83.

Bos, N., L. Grinsted, and L. Holman. 2011. Wax on, wax off: Nest soil facilitates indirect transfer of recognition cues between ant nestmates. PLoS One 6.

Bos, N., F. J. Guerrieri, and P. d'Ettorre. 2010. Significance of chemical recognition cues is context dependent in ants. Anim. Behav. 80:839–844.

Boulay, R., A. Hefetz, V. Soroker, and A. Lenoir. 2000. *Camponotus fellah* colony integration: worker individuality necessitates frequent hydrocarbon exchanges. Anim. Behav. 59:1127–1133.

Bradbury, J., and S. Vehrencamp. 2001. Principles of Animal Communication. 2nd ed. Sinauer Associates, Inc., Sunderland, Massachusetts.

Brian, M. 1975. Larval recognition by workers of the ant *Myrmica*. Anim. Behav. 23:745-756.

Buschinger, A. 2009. Social parasitism among ants: a review (Hymenoptera: Formicidae). Myrmecological News 12:219–235.

Buschinger, A., W. Ehrhardt, and U. Winter. 1980. The Organization of Slave Raids in Dulotic Ants - a Comparative Study (Hymenoptera; Formicidae). Z. Tierpsychol. 53:245–264.

Carlin, N. F., and P. H. Schwartz. 1989. Pre-imaginal experience and nestmate brood recognition in the carpenter ant, *Camponotus floridanus*. Anim. Behav. 38:89–95.

Chapuisat, M. 2004. Nestmate recognition in the unicolonial ant *Formica paralugubris*. Behav. Ecol. 16:15–19.

Chernenko, A., H. Helanterä, and L. Sundström. 2011. Egg recognition and social parasitism in *Formica* ants. Ethology 117:1081–1092.

Chernenko, A., M. Vidal-Garcia, H. Helanterä, and L. Sundström. 2013. Colony take-over and brood survival in temporary social parasite of the ant genus *Formica*. Behav Ecol Sociobiol 67:727–735.

Collingwood, C. A. 1979. The Formicidae (Hymenoptera) of Fennoscandia and Denmark. Scandinavian Science Press Ltd., Klampenborg, Denmark.

Crespi, B. 1992. Cannibalism and trophic eggs in subsocial and eusocial insects. Pp. 176–213 *in* M. Elgar and B. Crespi, eds. Cannibalism: ecology and evolution among diverce taxa. Oxford University Press, New York.

Crozier, R. H., and M. W. Dix. 1979. Analysis of two genetic models for the innate components of colony odor in social Hymenoptera. Behav. Ecol. Sociobiol. 4:217–224.

Cummings, D. L. D., G. J. Gamboa, and B. J. Harding. 1999. Lateral Vibrations by Social Wasps Signal Larvae to Withhold Salivary Secretions (*Polistes fuscatus*, Hymenoptera: Vespidae). J. Insect Behav. 12:465–473.

Czechowski, W., A. Radchenko, and W. Czechowska. 2002. Ants of poland : Museum & Institute Of Zoo.

d'Ettorre, P., and D. Hughes (eds). 2008. Sociobiology of communication: an interdisciplinary perspective. Oxford University Press, New York.

d'Ettorre, P., and A. Lenoir. 2011. Nestmate recognition. Pp. 194–208 *in* L. Lach, C. Parr, and K. Abbott, eds. Ant Ecology. Oxford University Press, USA.

de Sousa, R. 2008. Grades of communication. Pp. 275–288 *in* Sociobiology of Communication. Oxford University Press, New York.

Dietemann, V., J. Liebig, B. Hölldobler, and C. Peeters. 2005. Changes in the cuticular hydrocarbons of incipient reproductives correlate with triggering of worker policing in the bulldog ant *Myrmecia gulosa*. Behav. Ecol. Sociobiol. 58:486–496.

Downs, S. G., and F. L. W. Ratnieks. 2000. Adaptive shifts in honey bee (*Apis mellifera L.*) guarding behavior support predictions of the acceptance threshold model. Behav. Ecol. 11:326–333.

Edwards, J. P. 1991. Caste regulation in the pharaoh's ant *Monomorium pharaonis*: recognition and cannibalism of sexual brood by workers. Physiol. Entomol.16:263-271.

Elmes, G., T. Akino, J. Thomas, R. Clarke, and J. Knapp. 2002. Interspecific differences in cuticular hydrocarbon profiles of *Myrmica* ants are sufficiently consistent to explain host specificity by *Maculinea* (large blue) butterflies. Oecologia 130:525–535.

Fénéron, R., and P. Jaisson. 1995. Ontogeny of nestmate brood recognition in a primitive ant, *Ectatomma tubercalutum Olivier* (Ponerinae). Anim. Behav. 50:9–14.

Fouks, B., P. D'Ettorre, and V. Nehring. 2011. Brood adoption in the leaf-cutting ant *Acromyrmex echinatior*: adaptation or recognition noise? Insectes Soc. 58:479–485.

Fox, E. G. P., D. R. Solis, M. L. Rossi, W. Mackay, and J. Pacheco. 2011. Morphological notes on the worker and queen larvae of the thief ant *Solenopsis helena* (Hymenoptera, Formicidae, Myrmicinae) from Brazil. Florida Entomol. 94(4):909–915.

Franks, N. R., and A. B. Sendova-Franks. 1992. Brood sorting by ants: distributing the workload over the work-surface. Behav. Ecol. Sociobiol. 30:109–123.

Fujikawa, K., K. Seno, and M. Ozaki. 2006. A novel Takeout-like protein expressed in the taste and olfactory organs of the blowfly, Phormia regina. FEBS J. 273:4311–21.

Gamboa, G. J. 2004. Kin recognition in eusocial wasps. Ann. Zool. Fennici 41:789–808.

Gibbs, A. G. 1998. Water-Proofing Properties of Cuticular Lipids. Integr. Comp. Biol. 38:471–482.

Grüter, C., E. Jongepier, and S. Foitzik. 2017. Insect societies fight back: the evolution of defensive traits against social parasites. Phil. Trans. R. Soc. B 373 373:1–10.

Guerrieri, F. J., V. Nehring, C. G. Jorgensen, J. Nielsen, C. G.

Galizia, and P. D'Ettorre. 2009. Ants recognize foes and not friends. Proc. R. Soc. B Biol. Sci. 276:2461–2468.

Guerrieri, F., M. Schubert, J.-C. Sandoz, and M. Giurfa. 2005. Perceptual and Neural Olfactory Similarity in Honeybees. PLoS Biol. 3:e60.

Guntur, K. V. P., D. Velasquez, L. Chadwell, C. Carroll, S. Weintraub, J. A. Cassill, and R. Renthal. 2004. Apolipophorin-III-like protein expressed in the antenna of the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). Arch. Insect Biochem. Physiol. 57:101–110.

Hamilton, W. D. 1964. The genetical evolution of social behavior, parts 1 and 2. J. Theor. Biol. 1–52.

Hannonen, M., and L. Sundström. 2003. Sociobiology: Worker nepotism among polygynous ants. Nature 421:910.

Hare, J. F., and T. M. Alloway. 1987. Early learning and brood discrimination in leptothoracine ants (Hymenoptera: Formicidae). Anim. Behav. 35:1720–1724.

Helanterä, H., and P. d'Ettorre. 2014. A comparative study of egg recognition signature mixtures in *Formica* ants. Evolution 69:520–529.

Helanterä, H., Y. R. Lee, F. P. Drijfhout, and S. J. Martin. 2011. Genetic diversity, colony chemical phenotype, and nest mate recognition in the ant *Formica fusca*. Behav. Ecol. 22:710–716.

Helanterä, H., S. Martin, and F. Ratnieks. 2014. Recognition of nestmate eggs in the ant *Formica fusca* is based on queen derived cues. Curr. Zool. 60:131–136.

Helanterä, H., and F. L. W. Ratnieks. 2009. Two independent mechanisms of egg recognition in worker *Formica fusca* ants. Behav. Ecol. Sociobiol. 63:573–580.

Helanterä, H., and L. Sundström. 2007a. Worker policing and nest mate recognition in the ant *Formica fusca*. Behav. Ecol. Sociobiol. 61:1143–1149.

Helanterä, H., and L. Sundström. 2007b. Worker Reproduction in *Formica* Ants. Am. Nat. 170:E14–E25.

Helanterä, H., and L. Sundström. 2005. Worker reproduction in the ant *Formica fusca*. J. Evol. Biol. 18:162–171.

Hildebrand, J. G., and G. M. Shepherd. 1997. Mechanisms of olfactory discrimination:Converging Evidence for Common Principles Across Phyla. Annu. Rev. Neurosci. 20:595–631.

Hojo, M. K., K. Ishii, M. Sakura, K. Yamaguchi, S. Shigenobu, and M. Ozaki. 2015. Antennal RNA-sequencing analysis reveals evolutionary aspects of chemosensory proteins in the carpenter ant, *Camponotus japonicus*. Sci. Rep. 5:13541.

Holman, L., C. G. Jørgensen, J. Nielsen, and P. d'Ettorre. 2010. Identification of an ant queen pheromone regulating worker sterility. Proc. R. Soc. B Biol. Sci. 277:3793–3800.

Ishida, Y., V. Chiang, and W. S. Leal. 2002. Protein that makes sense in the Argentine ant. Naturwissenschaften 89:505–507.

Isingrini, M., A. Lenoir, and P. Jaisson. 1985. Preimaginal learning as a basis of colony-brood recognition in the ant *Cataglyphis cursor*. Proc. Natl. Acad. Sci. U. S. A. 82:8545–8547.

Jaisson, P. 1991. Kin Recognition. Cambridge University Press, Cambridge.

Johnson, C. A., H. Topoff, R. K. Vander Meer, and B. Lavine. 2005. Do these eggs smell funny to you?: an experimental study of egg discrimination by hosts of the social parasite *Polyergus breviceps* (Hymenoptera: Formicidae). Behav. Ecol. Sociobiol. 57:245–255.

Katzav-Gozansky, T., R. Boulay, R. Vander Meer, and A. Hefetz. 2004. In-nest environment modulates nestmate recognition in the ant *Camponotus fellah*. Naturwissenschaften 91:186–190.

Kleeberg, I., F. Menzel, and S. Foitzik. 2017. The influence of slavemaking lifestyle, caste and sex on chemical profiles in *Temnothorax* ants: insights into the evolution of cuticular hydrocarbons. Proc. R. Soc. B Biol. Sci. 284:20162249.

Kulmuni, J., and H. Havukainen. 2013. Insights into the evolution of the CSP gene family through the integration of evolutionary analysis and comparative protein modeling. PLoS One 8:e63688.

Leboeuf, A. C., P. Waridel, C. S. Brent, A. N. Gonç Alves, L. Menin, D. Ortiz, O. Riba-Grognuz, A. Koto, Z. G. Soares, E. Privman, E. A. Miska, R. Benton, and L. Keller. 2016. Oral transfer of chemical cues, growth proteins and hormones in social insects. doi: 10.7554/eLife.20375.001.

Lenoir, A. 1981. Brood retrieving in the ant, *Lasius niger L.* Sociobiology 6:153–178.

Lenoir, A., D. Fresneau, C. Errard, and A. Hefetz. 1999. Individuality and colonial identity in ants: the emergence of the social representation concept. Pp. 219–237 *in* Information Processing in Social Insects. Birkhäuser Basel, Basel.

Leonhardt, S. D., A. S. Brandstaetter, and C. J. Kleineidam. 2007. Reformation process of the neuronal template for nestmate-recognition cues in the carpenter ant *Camponotus floridanus*. J. Comp. Physiol. A 193:993–1000.

Liebert, A. E., and P. T. Starks. 2004. The action component of recognition systems: A focus on the response. Ann. Zool. Fennici 41:747–764.

Maeder, A., A. Freitag, and D. Cherix. 2005. Species- and nestmate brood discrimination in the sibling wood ant species *Formica paralugubris* and *Formica lugubris*. Ann. Zool. Fennici 42:201–212.

Martin, S., and F. Drijfhout. 2009a. A review of ant cuticular hydrocarbons. J. Chem. Ecol. 35:1151–61.

Martin, S. J., and F. P. Drijfhout. 2009b. Nestmate and task cues are influenced and encoded differently within ant cuticular hydrocarbon profiles. J. Chem. Ecol. 35:368–74.

Martin, S. J., H. Helanterä, and F. P. Drijfhout. 2011. Is parasite pressure a driver of chemical cue diversity in ants? Proc. Biol. Sci. 278:496–503.

Martin, S. J., S. Shemilt, and F. P. Drijfhout. 2012a. Effect of time on colony odour stability in the ant *Formica exsecta*. Naturwissenschaften 99:327–31.

Martin, S. J., E. Vitikainen, F. P. Drijfhout, and D. Jackson. 2012b. Conspecific ant aggression is correlated with chemical distance, but not with genetic or spatial distance. Behav. Genet. 42:323–31.

Martin, S. J., E. Vitikainen, H. Helanterä, and F. P. Drijfhout. 2008. Chemical basis of nest-mate discrimination in the ant *Formica exsecta*. Proc. Biol. Sci. 275:1271–8.

Martin, S. J., E. Vitikainen, S. Shemilt, F. P. Drijfhout, and L. Sundström. 2013. Sources of variation in cuticular hydrocarbons in the ant *Formica exsecta*. J. Chem. Ecol. 39:1415–23.

Mateo, J. M. 2004. Recognition systems and biological organization: The perception component of social recognition. Ann. Zool. Fennici 41:729–745.

McKenzie, S. K., P. R. Oxley, and D. J. Kronauer. 2014. Comparative genomics and transcriptomics in ants provide new insights into the evolution and function of odorant binding and chemosensory proteins. BMC Genomics 15:718. BioMed Central.

Nehring, V., S. E. F. Evison, L. A. Santorelli, P. d'Ettorre, and W. O. H. Hughes. 2011. Kin-informative recognition cues in ants. Proc. Biol. Sci. 278:1942–8.

Orivel, J., C. Errard, and A. Dejean. 1997. Ant gardens: interspecific recognition in parabiotic ant species. Behav Ecol Sociobiol 40:87–93.

Ozaki, M., and A. Wada-Katsumata. 2010. Perception and olfaction of cuticular compounds. Pp. 207–221 *in* G. Blomqvist and A. G. Bagnères, eds. Insect Hydrocarbons: Biology, Biochemistry and Chemical Ecology. Cambridge University Press, Cambridge.

Ozaki, M., A. Wada-Katsumata, K. Fujikawa, M. Iwasaki, F. Yokohari, Y. Satoji, T. Nisimura, and R. Yamaoka. 2005. Ant nestmate and non-nestmate discrimination by a chemosensory sensillum. Science 309:311–4.

Peignier, M., T. Pokorny, J. Heinze, R. Lindgren, H. Helanterä, and E. Schultner. (2019). Honesty of larval begging signals is shaped by colony kin structure in *Formica* ants. Front. Ecol. Evol. 7:398.

Penick, C. A., and J. Liebig. 2017. A larval 'princess pheromone' identifies future ant queens based on their juvenile hormone content. Anim. Behav. 128:33–40.

Pulliainen, U., H. Helanterä, L. Sundström, and E. Schultner. 2019. The possible role of ant larvae in the defence against social parasites. Proc. - R. Soc. London, B 286: 20182.

Punttila, P., and Y. Haila. 1996. Colonisation of a burned forest by ants in the Southern Finnish boreal forest. Silva Fenn. 30:421–435.

Punttila, P., Y. Haila, T. Pajunen, and H. Tukia. 1991. Colonisation of Clearcut Forests by Ants in the Southern Finnish Taiga: A Quantitative Survey. Oikos 61:250–262.

Reeve, H. 1989. The evolution of conspecific acceptance thresholds. Am. Nat. 133:407–435.

Richard, F.-J., M. Poulsen, F. Drijfhout, G. Jones, and J. J. Boomsma. 2007. Specificity in Chemical Profiles of Workers, Brood and Mutualistic Fungi in *Atta, Acromyrmex*, and *Sericomyrmex* Fungus-growing Ants. J. Chem. Ecol. 33:2281–2292.

Rosengren, R., and D. Cherix. 1981. The Pupa-Carrying Test as a Taxonomic Tool in the *Formica rufa* Group. Pp. 263–281 *in* P. E. Howse and J.-L. Clement, eds. Biosystematics of social insects. Academic Press, London and New York.

Schmid-Hempel, P. 1995. Parasites and social insects. Princeton University Press, New Jersey.

Schultner, E., P. D'Ettorre, and H. Helanterä. 2013. Social conflict in ant larvae: egg cannibalism occurs mainly in males and larvae prefer alien eggs. Behav. Ecol. 24:1306–1311.

Schultner, E., A. Gardner, M. Karhunen, and H. Helanterä. 2014. Ant larvae as players in social conflict: relatedness and individual identity mediate cannibalism intensity. Am. Nat. 184:E161-74.

Schultner, E., J. Oettler, and H. Helanterä. 2017. The Role of Brood in Eusocial Hymenoptera. Q. Rev. Biol. 92:39–78.

Schultner, E., and U. Pulliainen. (2019) Brood recognition and discrimination in ants. Accepted for publication.

Seifert, B. 2007. Die Ameisen Mittel- und Nordeuropas. Lutra-Verl.-und-Vertriebsges. Görlitz, Tauer.

Sevala, V. L., A.-G. Bagnères, M. Kuenzli, G. J. Blomquist, and C. Schal. 2000. Cuticular Hydrocarbons of the Dampwood Termite, *Zootermopsis nevadensis*: Caste Differences and Role of Lipophorin in Transport of Hydrocarbons and Hydrocarbon Metabolites. J. Chem. Ecol. 26:765–789.

Sherman, P. ., H. . Reeve, and D. . Pfennig. 1997. Recognition systems. Pp. 69–96 *in* N. B. D. J.R. Krebs, ed. Behavioural Ecology: an Evolutionary Approach. Blackwell Scientific, Oxford.

Signorotti, L., P. Jaisson, and P. d'Ettorre. 2013. Larval memory affects adult nest-mate recognition in the ant *Aphaenogaster senilis*. Proc. R. Soc. B Biol. Sci. 281:20132579–20132579.

Soroker, V., and A. Hefetz. 2000. Hydrocarbon site of synthesis and circulation in the desert ant *Cataglyphis niger*. J. Insect Physiol. 46:1097–1102.

Souza, D. J., T. M. C. Della Lucia, C. Errard, F.-J. Richard, and E. R. Lima. 2006. Behavioural and chemical studies of discrimination processes in the leaf-cutting ant *Acromyrmex laticeps nigrosetosus* (Forel, 1908). Brazilian J. Biol. Rev. brasleira Biol. 66:863–871.

Sturgis, S. J., and D. M. Gordon. 2012. Nestmate recognition in ants (Hymenoptera: Formicidae): a review. Myrmecological News 16:101–110.

Sundström, L., M. Chapuisat, and L. Keller. 1996. Conditional Manipulation of Sex Ratios by Ant Workers: A Test of Kin Selection Theory. Science 274:993–995.

Tsutsui, N. D. 2013. Dissecting ant recognition systems in the age of genomics. Biol. Lett. 9:20130416–20130416.

Urbani, C. B. 1991. Indiscriminate oophagy by ant larvae: an explanation for brood serial organization? Insectes Soc. 38:229–239.

van Zweden, J. S., J. B. Brask, J. H. Christensen, J. J. Boomsma, T. A. Linksvayer, and P. D'Ettorre. 2010. Blending of heritable recognition cues among ant nestmates creates distinct colony gestalt odours but prevents within-colony nepotism. J. Evol. Biol. 23:1498–1508.

van Zweden, J. S., and P. d'Ettorre. 2010. Nestmate recognition in social insects and the role of hydrocarbons. P. *in* G. J. Blomquist and A. Bagneres, eds. Insect Hydrocarbons: Biology, Biochemistry and Chemical Ecology. Cambridge University Press, Cambridge.

Viana, A. M. M., A. Frézard, C. Malosse, T. M. C. Della Lucia, C. Errard, and A. Lenoir. 2001. Colonial recognition of fungus in the fungus-growing ant *Acromyrmex subterraneus subterraneus* (Hymenoptera: Formicidae). Chemoecology 11:29–36.

Villalta, I., F. Amor, X. Cerdá, and R. Boulay. 2016. Social coercion of larval development in an ant species. Sci. Nat. 103:18.

Vitikainen, E., C. Haag-Liautard, and L. Sundström. 2011. Inbreeding and reproductive investment in the ant *Formica exsecta*. Evolution 65:2026–37.

Wagner, D., M. J. F. Brown, P. Broun, W. Cuevas, L. E. Moses, D. L. Chao, and D. M. Gordon. 1998. Task-Related Differences in the Cuticular Hydrocarbon Composition of Harvester Ants, *Pogonomyrmex barbatus*. J. Chem. Ecol. 24:2021–2037.

Wicker-Thomas, C., and T. Chertemps. 2010. Molecular biology and genetics of hydrocarbon production. Pp. 53–74 *in* G. J. Blomquist and A.-G. Bagneres, eds. Insect Hydrocarbons: Biology, Biochemistry and Chemical Ecology. Cambridge University Press, Cambridge.

Willingham, A. T., and T. Keil. 2004. A tissue specific cytochrome P450 required for the structure and function of *Drosophila* sensory organs. Mech. Dev. 121:1289–1297.

Wilson, E. O. 1971. The insect societies. Belknap Press of Harward Univ. Press, Cambridge, MA.

Wyatt, T. D. 2003. Pheromones and Animal Behaviour Communication by Smell and Taste. Cambridge University Press, Cambridge.

Wyatt, T. D. 2010. Pheromones and signature mixtures: defining species-wide signals and variable cues for identity in both invertebrates and vertebrates. J. Comp. Physiol. A. Neuroethol. Sens. Neural. Behav. Physiol. 196:685–700.

Yoshizawa, Y., R. Sato, K. Tsuchihara, K. Ozaki, K. Mita, K. Asaoka, and K. Taniai. 2011. Ligand carrier protein genes expressed in larval chemosensory organs of *Bombyx mori*. Insect Biochem. Mol. Biol. 41:545–562.